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SIMULTANEOUS DETERMINATION OF ARTEMISININ AND ITS BIOPRECURSORS IN *ARTEMISIA ANNUA*

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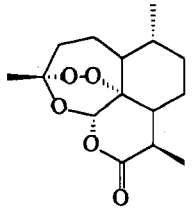
ABSTRACT.—*Artemisia annua* has been used for the treatment of fever and malaria. Artemisinin [**1**], a sesquiterpene lactone constituent of this plant, is responsible for its therapeutic effects. A rapid, sensitive, and specific reversed-phase hplc method using electrochemical and uv detection has been developed for the simultaneous determination in plant extracts of **1** and its bioprecursors artemisinic acid [**2**], arteannuin B [**3**], and artemisitene [**4**].

Malaria continues to be a major health problem in many areas of the world. The World Health Organization reports that some 300 million people are believed to be infected with malaria parasites, with 90% of them living in tropical Africa. The control of malaria is becoming more difficult due to the increased resistance of *Plasmodium* strains to commonly used drugs such as chloroquine and mefloquine. Hence, there is an interest in traditional medicinal plants that have been used to cure fever and malaria.

Artemisia annua L. is an herb from the Compositae family, used for many centuries in Chinese traditional medicine for the treatment of fever and malaria (1). Artemisinin ("qinghaosu"), an endoperoxide-containing sesquiterpene lactone [**1**], is known to be responsible for the therapeutic effects of the plant (2). Its efficiency was demonstrated against chloroquine-resistant strains of *Plasmodium* parasites and cerebral malaria (3). The most widely used derivatives of **1** are artemether [**5**], arteether, and artesunate, which are the methyl ether, ethyl ether, and hemisuccinate ester of dihydroartemisinin, respectively (3,4). Such derivatives enhance drug delivery, are eight to ten times more potent as **1**, and can only be obtained commercially from the parent compound. Several total syntheses of **1** have been reported (5), but the chemical nature of the compound is complex and therefore this drug cannot be synthesized chemically in an economically feasible way. The *A. annua* plant itself is, therefore, the only practical source of this valuable drug. The goal of our research program is to increase the concentration of **1** in *A. annua* through genetic engineering. However, we consider that it is first necessary to obtain further insight into the enzymatic processes governing the biosynthetic pathway of **1** in



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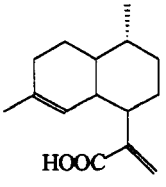

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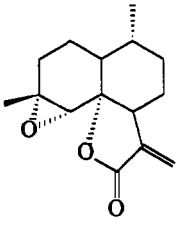

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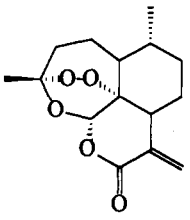

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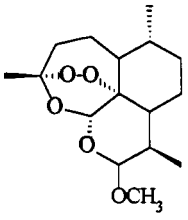

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order to be able to influence this pathway. Preliminary results have already been obtained for the transformation of *A. annua* plants (A.N. Vergauwe, University of Ghent, unpublished results).

The fundamental precursor of the sesquiterpenes, farnesyl pyrophosphate, is synthesized from mevalonic acid (6). Farnesyl pyrophosphate can cyclize to a germacrane and a cadinane skeleton, among many others. After oxidation, artemisinic acid (arteannuic acid) [**2**] is formed (7). This intermediate in the proposed biosynthetic pathway of **1** may be converted into arteannuin B [**3**] (6,8). Nair and Basile (9) suggested a hypothetical bioconversion pathway of **3** to **1**. Artemisitene [**4**] is very closely related to **1** and has been reported as being converted into **1** (9,10). An analytical method which would allow the quantitative determination of both **1** and its bioprecursors is necessary in order to study the content of **1** in transgenic plants.

Several methods have been used for the detection of **1** and its synthetic derivatives, namely, hplc (11–17), radioisotopic labeling (18), radioimmunoassay (19), ELISA (20), colorometric determination (21), tlc (22), gc (23), and gc-ms (9,24). Hplc with uv detection of **1** requires derivatization with NaOH (11). However, hplc using electrochemical detection (hplc-ec) requires no derivatization of **1** and is based on the reduction of its endoperoxide (12,13). A gc-ms method has been reported for the simultaneous determination of **1–3** (9), and recently Ferreira *et al.* (25) determined **1** and **4** by hplc-ec, but no detectable amounts of **4** were found in crude plant extracts.

No simultaneous and simple detection and quantification method of **1** and its important bioprecursors exists in the literature, however. The present work reports an hplc method combining ec with uv detection, so compounds **1–4** can be detected simultaneously, and should constitute a useful tool in the investigation of the biosynthetic pathway of **1**.

Artemisinin [**1**] is uv-transparent but the endoperoxide bridge can undergo electrochemical reduction (12,13). Based on this property, an hplc procedure with ec detection has been developed for plant material using solid-phase extraction as a clean-up of the plant extract.

Artemisitene [**4**] can be determined using uv detection but the more sensitive ec detection, based on the endoperoxide, is preferred. Because molecular oxygen is also reduced, special precautions were required to deoxygenate the hplc system, including the injected sample using a modified injector. A sample, injected without degassing, gave rise to a baseline destabilization of more than 50 min, probably due to an oxygen saturation at the ec detector. The other precursors, compounds **1–3**, were detected by uv absorption since they lack an endoperoxide functionality. A wavelength of 228 nm was determined as the best compromise for the detection of **2** and **3**.

The ec and uv detection were performed simultaneously so that **1** and its precursors from plant extracts could be determined in one elution. Chromatograms of a mixture of **1** and its bioprecursors and of an *A. annua* leaf extract are shown in Figure 1. All compounds were clearly resolved. The identification of the peaks in the leaf extract was performed by comparison to known reference standards using solvent systems with different ratios of NaOAc buffer and CH_3CN.

The use of hexane (26), toluene (27), or petroleum ether (40–65°) (1) as potential extraction solvents was compared. Extraction with toluene and hexane resulted in a comparably high recovery. Toluene was chosen since two extractions with 3 ml were sufficient to extract all of the compounds of interest. No statistically significant differences have been found between our rapid extraction method in a sonicator and the 48-h long

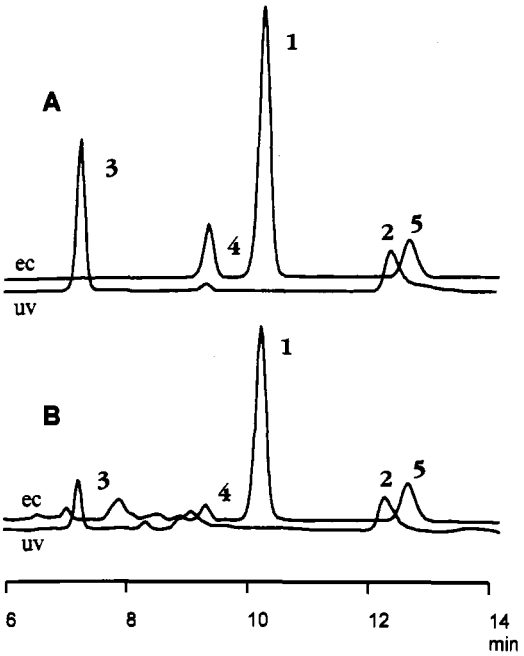

FIGURE 1. Chromatograms of a standard solution (A) (50 μl/ml arteannuin B, 2 μg/ml artemisitene, 10 μg/ml artemisinin, 50 μg/ml artemisinic acid, 5 μg/ml artemether) and a plant extract (B) with 5 μg/ml artemether as internal standard. Run time=14 min; **1**=artemisinin; **2**=artemisinic acid; **3**=arteannuin B; **4**=artemisitene; **5**=artemether.

extraction method reported by Charles *et al.* (28).

In order to clean up the plant extract, a normal-phase Si gel solid-phase extraction method was developed. The combination of normal-phase solid-phase extraction with reversed-phase hplc has the advantage that relatively apolar compounds can be eluted from the solid-phase extraction column, so that these compounds do not disturb successive hplc elutions.

Standard curves and control standards were prepared by spiking an EtOH-H_2O (50:50) solution or the extraction solvent with **1–4** and **5** (internal standard) in concentrations from 0.2 to 50 μg/ml. The linearity (coefficient of correlation) of the hplc method was 0.9999 for **3** in a concentration range of 1–50 μg/ml, 0.9998 for **4** in a range of 0.2–2 μg/ml, 0.9997 for **1** in a range of 0.2–1 μg/ml, and 0.9999 for **2** in a range of 1–50 μg/ml. After solid-phase extraction, the coefficient of correlation of a standard curve in a concentration range from 0.5–7 μg/ml of **1** was 0.9994. The diurnal variability of the hplc method was determined for the different compounds in a concentration comparable to that found in plants. The coefficients of variation for **1–4** were 0.96%, 4.59%, 1.42%, and 1.58%, respectively (n=13). When using the combined solid-phase extraction and hplc method, the diurnal variability of **1** was 7.2% (n=10). The inter-day variability of the solid-phase extraction and hplc method was 13.4% (n=20).

With a 20-μl loop, the detection limits for the ec detector were 7.5 ng/ml for **4** and 10 mg/ml for **1**. The uv detector had detection limits of 250 ng/ml for **3**, 200 ng/ml for **4**, and 1000 ng/ml for **2**. With these detection limits, no detection

problems occurred since the naturally occurring concentrations in the plant are well above these limits. A 100% recovery of **1** was found after solid-phase extraction.

The content of artemisinin [**1**] in plant leaves, using this method, varied between 0.06% and 0.16% dry wt which is comparable with the results reported by Liersch *et al.* (29). The content of compounds **1–4** in the leaves of 6 plants after 3 months was 0.071±0.008%, 0.41±0.04%, 0.1101±0.0001%, and 0.008±0.001% dry wt, respectively (Figure 2).

In the present report, hplc conditions have been developed for the simultaneous determination of **1** and its bioprecursors **2–4** in plant extracts after sample clean up with Si gel solid-phase extraction. This methodology enables the quantitative determination of **1** as well as its bioprecursors during the development and growth of *A. annua*.

EXPERIMENTAL

GENERAL EXPERIMENTAL CONDITIONS.—Hplc with reductive electrochemical and uv detection was performed with a model BAS 200a chromatograph (Bioanalytical Systems, West Lafayette, IN). The system was equipped with three mobile phase reservoirs, a dual piston pump, a Zorbax SB-CN column (4.6 mm×25 cm, Rockland Technologies, ATAS, Veldhoven, The Netherlands), a dual thin-layer electrode with a Ag/AgCl reference electrode, a uv detector, and a Rheodyne injector, which was modified because the sample requires degassing prior to injection. The ec detection was performed with a glassy carbon electrode run in parallel mode at a potential of −1.0 V versus Ag/AgCl (14). The hplc system recycles the mobile phase in a closed circuit and was equipped for mobile phase heating and sparging. Stainless steel connectors and tubing were used to prevent oxygen entering the system.

The premixed mobile phase consisted of CH_3CN-0.1 M NaOAc (pH 5) (40:60), and the flow rate was 1.5 ml/min. The system was operated at the following temperatures: mobile phase, 35°, column oven and cell, 31°. The uv detector was set at 228 nm.

Freshly prepared mobile phase was rigorously deoxygenated with He at a high flow rate for 30 min at 35° to remove dissolved oxygen. A pressure head of He was maintained on the mobile phase. When retention times became excessively long due to preferential evaporation of CH_3CN, a new mobile phase was used. Under these condi-

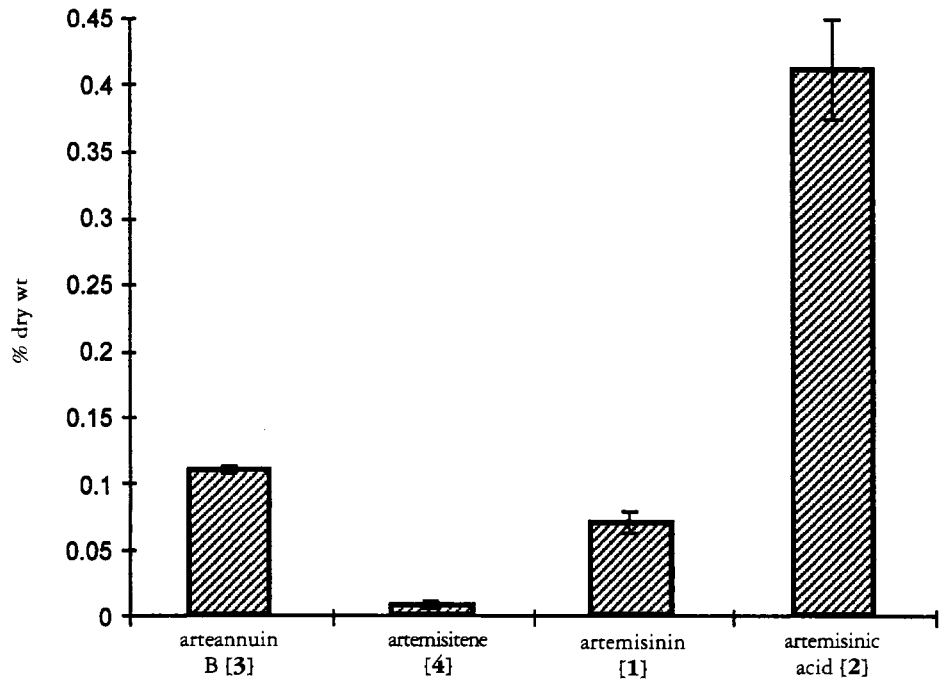

FIGURE 2. Content (% dry wt) of artemisinin [**1**] and its bioprecursors in the leaves of *A. annua* after 3 months (n=6).

tions, background currents were in the range 170–250 nA. The samples were sparged for 12 min with He prior to injection. All glassware was silanized before use with a 1% aqueous solution (v/v) of Pierce AquaSil (Rockford, IL) according to the manufacturer's recommendations. Purified artemisinin [**1**], artemisinic acid [**2**], arteannuin B [**3**], and artemisitene [**4**] were provided by the late Dr. D.L. Klayman (Walter Reed Army Institute of Research, Washington, DC). Artemether [**5**] was provided by Dragon Pharmaceuticals (Owmbran Gwent, England). All solvents used were of hplc grade.

PLANT MATERIAL.—Seeds of *A. annua* from the former Yugoslavia were donated by the Walter Reed Army Institute of Research, Washington, DC. Plants were cultivated in an experimental greenhouse using Hg and Na vapor lamps, 16 h/day, at a temperature of 22° and a relative humidity of 40%. Only the leaves were used for extraction since they contain 89% of the total **1** in the plant (28). Leaves were collected before flower buds were visible.

EXTRACTION AND ANALYSIS.—About 100 mg of lyophilized leaf powder were accurately weighed and extracted for 30 min in a sonicator with 3 ml toluene containing **5** as internal standard. After centrifugation (4 min, 4000 rpm) the solvent was decanted. This procedure was repeated with another 3 ml of extraction solvent. After centrifugation the solvent was again decanted and added to the first 3 ml. A 1-ml aliquot of the combined extracts was used in the solid-phase extraction.

A normal-phase 500 mg Si gel solid-phase extraction column (Varian, Harbor City, CA) was preconditioned with 3 ml of toluene. An aliquot of 1 ml of the plant extract was passed through the column, followed by washing with 2 ml petroleum ether-Et_2O (9:1); the eluates were discarded. Lower concentrations of petroleum ether led to losses of **5**. Compounds **1–4** were eluted with 2×0.5 ml MeCN. The eluate was evaporated under N_2 to dryness and reconstituted in 1 ml EtOH-H_2O (50:50). A 400-µl aliquot of the sample was filtered through a 0.45-µm syringe filter (Schleicher & Schuell, Dassel, Germany) prior to degassing and injection.

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